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International application number: PCT/EP05/000934

International filing date: 31 January 2005 (31.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: GB
Number: 0402051.7
Filing date: 29 January 2004 (29.01.2004)

Date of receipt at the International Bureau: 05 April 2005 (05.04.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

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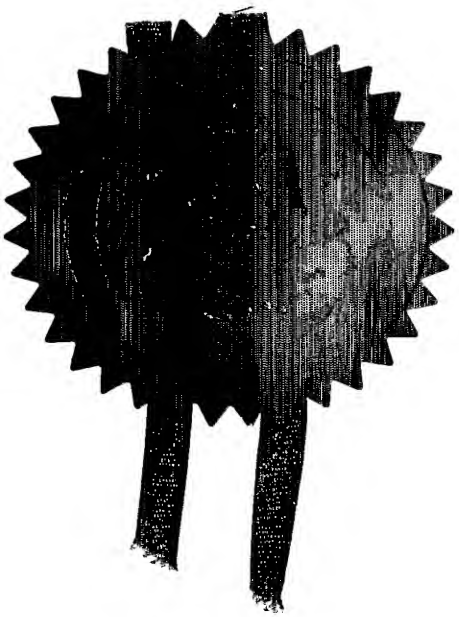

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P-312

The stem cell gene *axot* is associated with regulation of LIF and mitogenic activation of T lymphocytes.

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Control of "stemness"[1] for self-renewal of stem cells, *versus* their differentiation during organogenesis, is fundamental to the new field of regenerative medicine. Leukaemia inhibitory factor (LIF) is critical to this control, acting as a suppressor of stem cell differentiation[2,3]. The discovery that both LIF and *axot*, a novel stem cell gene[1,4], are linked also to immune tolerance (S.M.M. et al, manuscript submitted) suggests a relationship between stemness and immunity. To explore this relationship we have asked if immune cells from *axot*$^{-/-}$ mice differ from those of *axot*$^{+/+}$ littermates. We discovered (i) that presence of axotrophin is involved in damping down proliferation of T, but not B, lymphocytes; (ii) that lack of axotrophin leads to excessive release of T cell cytokines; and (iii) an *axot* gene-dose dependent suppression of LIF. This is the first evidence that fate determination mediated by LIF maybe linked to axotrophin, and demonstrates commonalities between stemness and immune tolerance that may favour acceptance of implanted stem cell allo-grafts for therapeutic tissue regeneration.

Fate determination in stem cells is a critical feature in development, providing a balance between pluripotent self-renewal *versus* differentiated function within the whole organism. In regenerative medicine, understanding the molecular basis of fate determination of stem cells is important if they are to be used successfully in the treatment of disease. Fate determination pathways also play a key role in the immune system, where reactivity is finely tuned to ensure protective tolerance towards self tissues whilst simultaneously being capable of aggressive attack towards foreign pathogens. Although the regulatory tolerance pathway is little understood, the recent demonstration that a single gene, *foxp3*, is able to orchestrate the differentiation of naïve CD4+ T cells into regulatory T cells (Treg)[5,6,7] implies the existence of "master" switches for fate determination in immunity. We have recently discovered features of immune tolerance that are common to regulation of stem cell fate, raising two important questions: do "stemness" signals play a role in autoimmunity by suppressing terminal differentiation of immune effector cells? Do allogeneic stem cells bias the allo-immune response towards allo-tolerance, by signalling for "stemness", so favouring successful therapeutic engraftment? This paper describes how we discovered that axotrophin, expressed in embryonic, neuronal, and haematopoietic stem cells[1], is not only involved in regulation of T lymphocyte reactivity, but also in regulation of LIF, thereby providing a novel concept of immunoregulation.

The molecular events associated with immune tolerance, *versus* immune aggression, have been compared in previous studies using an *ex vivo* model[8]. This is derived from mice where fully mismatched heart grafts, normally rejected by day 7, become accepted indefinitely after short term blockade of CD4 and CD8 (ref. 9). Once established, this transplantation tolerance is self-perpetuating and isolated "tolerant" spleen cells show powerful immune regulatory properties, being able to impose donor-specific allo-tolerance when infused into fully immune competent naïve recipients. We characterised the *ex vivo* responses of the tolerant spleen cells, *versus* spleen cells from mice that had been primed to reject the same donor-type (S.M.M. et al, manuscripts submitted) and the key features of rejection were rapid interferon gamma (IFNγ) release and strongly amplified expression of genes encoding IFNγ and granzyme B. In marked contrast, tolerance

showed features in common with stemness, these being the release of LIF and increases in c-kit (the receptor for stem cell factor (SCF)) and in STAT3 (signal transducer and activator of transcription 3, responsive to both SCF and LIF activity). We found that the relationship between LIF and tolerance was also evident in cloned Treg, showing high levels of LIF release in contrast to Th1 and Th2 clones. At the gene level, tolerance was associated with strong induction of a newly discovered stem cell gene, *axot* (Genbank accession number AF155739). *Axot* encodes axotrophin and *axot* null mice show abnormal neural development together with sensory neuron loss (M.A.H *et al.*, manuscript submitted). To test of our hypothesis that stemness and tolerance are linked, we have asked if axotrophin influences immune responsiveness.

We first looked at lymphocyte responsiveness to mitogen. *Axot* null ($axot^{-/-}$) mice were compared to littermates that expressed either one *axot* allele (heterozygous, $axot^{+/-}$) or both alleles (wild-type; $axot^{+/+}$). Whole cell populations were freshly isolated from the spleen and we measured mitogenic activation using either concanavalin A (conA) as a T cell mitogen, or lipopolysaccharide (LPS) as a B cell mitogen. We also looked for any kinetic effects on responsiveness by comparing DNA synthesis at 48h and at 72h. Since activated lymphocytes show a synchronised entry into the cell cycle, with S phase peaking at 48h (ref. 10), we reasoned that a consistent reduction in DNA synthesis in the *axot* null cells, compared to the $axot^{+/+}$ cells, would indicate a loss of mitogenic responsiveness due to lack of axotrophin. However, the level of T cell proliferation showed a marked increase in the *axot* null cells when compared to wild-type cells. This was not caused by altered kinetics since the *axot*-related differentials were similar at both 48h and 72h (Fig. 1*a*, Fig. 1*b*). Therefore axotrophin appeared to be **repressing** the proliferative response of T cells. Moreover, since the heterozygous $axot^{+/-}$ T cells showed intermediate hyper-proliferation, the repression appeared sensitive to *axot* gene dose. In marked contrast to the T cells, B lymphocyte proliferation was not significantly altered by axotrophin (Fig. 1*c*, Fig. 1*d*). We concluded that axotrophin plays a role in damping down T, but not B, lymphocyte proliferation following mitogenic stimulation. No spontaneous mitogenesis occurred in cultures of $axot^{+/+}$, $axot^{+/-}$, or $axot^{-/-}$ spleen cells over a 7d period (data not shown).

As a further test of functionality in the *axot* null spleen cells, we measured cytokine release in response to mitogen. Lack of axotrophin was associated with a two-fold increase in interleukin 2 (IL2) following conA treatment, in both *axot* null and *axot* heterozygous cell cultures (Fig. 2*a*). This IL2 equivalence revealed that IL2 was not a limiting factor for T cell proliferation, where there had been a four-fold difference. Splenic B cells did not release IL2 (Fig. 2*b*) whilst both T and B cells released IL10 in response to their respective mitogens. Again, only the conA-treated cultures were affected by a lack of axotrophin, with a ten-fold increase in IL10 in both $axot^{+/-}$ and $axot^{-/-}$ cell cultures (Fig. 2*c*, Fig. 2*d*). These findings show that partial or total reduction of axotrophin results in a general increment in both IL2 and IL10 from activated T cells, but has no effect on IL10 release from activated B cells. INFγ and IL4 were also measured and showed a similar *axot*-related increment to that found for IL2 in the conA-treated cultures, as detailed in the legend to Fig 1. LPS-treated cultures were negative for INFγ and IL4.

Unexpectedly, we found that release of LIF in response to conA was strongly inhibited by axotrophin and that this inhibition was gene-dose dependent (Fig. 2). There was no LIF in the LPS-treated cultures irrespective of *axot* genotype. Based on the relationship between LIF concentration *versus axot* gene dose, we have hypothesised that gene dose correlates with expression levels of axotrophin. Both LIF release and T cell proliferation would thus appear to be critically influenced by axotrophin and our results would be in accord with inter-dependent links between the three.

By analysis of phenotype and of histological structure, we looked for effects of axotrophin on the phenotypic composition of lymphoid organs. Cell sub-populations were identified by FACS analysis as follows: cells expressing the T cell markers CD3, CD4 and CD8; the B cell marker CD19; the activation marker of T cells and of regulatory tolerant T cells, CD25; and markers of dendritic cells, CD205 and DC33D1. None of these markers showed differential expression between the *axot*$^{+/+}$, *axot*$^{+/-}$, and *axot*$^{-/-}$ littermates (Fig. 3). Similarly, histological assessment of the spleen and thymus showed no significant differences between the three *axot* genotypes (data not shown).

Fate determination is controlled by genetic programmes that are altered by changing the nature and frequency of cytokine interactions within the microenvironment, both for totipotent and pluripotent stem cells, and for the differentiation of precursor cells. LIF is a key determinant of self-renewal of stem cells[11] in addition to being a neuropoietic cytokine[12]. Having shown that axotrophin may act as a negative regulator of LIF, at least in activated T cells, we suggest that LIF expression is functionally coupled to axotrophin expression, with axotrophin playing a role in co-ordinating the positive and negative regulation of LIF release. This would place axotrophin as a potential regulator of fate determination *via* LIF. The molecular function of axotrophin has yet to be determined and how axotrophin might influence LIF release is unknown. Future work will include exploration of this relationship, looking for effects of axotrophin on LIF gene expression[13], and on regulation of LIF-induced signalling through the LIF-R/gp130 complex[14,15,16,17].

As a working model we propose that LIF activity, regulated by axotrophin, is associated with immune tolerance. LIF may guide naïve T cells towards a relatively undifferentiated, non-aggressive phenotype in response to presented antigen, where the circumstances of presentation initiate the tolerogenic LIF activity, either directly or indirectly (eg antigen presentation by immature or regulatory dendritic cells[18,19] and associated vitamin D activity[20]; or reduced T cell responsiveness due to altered function of CD4/CD8 (ref. 9) or CD28 (ref. 21)). Thereafter, epigenetic changes, including expression of *foxp3* and *ROG*[18], and induction of Id transcription factors[22], would stabilise the tolerant phenotype for inheritable Treg activity. A link between stem cell biology and regulatory immune tolerance has direct relevance to therapeutic intervention of immune-related diseases and to immunosuppressive treatment of organ transplant recipients. The work also has major implications for use of stem cells for regenerative medicine, since the properties we have discovered may enhance successful outcome of implanted stem cells in patients.

In summary, we have discovered that axotrophin represses T lymphocyte proliferative responsiveness in adult mice and that axotrophin is able to act as a negative regulator of LIF, implying that axotrophin acts through LIF to regulate T cells.

METHODS

Mice

Gene trap insertion was used to generate *axot* null BALB/c mice and littermates from heterozygous parents were genotyped by PCR analysis of genomic DNA to identify *axot*$^{+/+}$, *axot*$^{+/-}$, and *axot*$^{-/-}$ pups as detailed previously (M.E.H. *et al*, manuscript submitted). Spleen, thymus and lymph node were obtained from 5m old littermates and kept on ice prior to cell preparation for the analyses described below. The lymph node tissue yielded very few cells and was discarded. Spleen and thymus from *axot*$^{+/+}$, *axot*$^{+/-}$, and *axot*$^{-/-}$ littermates were also taken for histology. These were bisected and fixed in 70% ethanol. Fixed tissues were embedded in paraffin blocks and sectioned, then stained with haematoxylin and eosin using standard procedures.

Proliferation assays

Splenocytes and thymocytes were teased out from each organ and collected in sterile growth medium [RPMI-1640 (Gibco™ Invitrogen Co.) supplemented with 10% FCS (Gibco™ Invitrogen Co.), 200mM L-Glutamine, 100U/mL Penicillin and 100µg/mL Streptomycin (Sigma Chemical Co.)]. The cell suspensions were washed, resuspended in growth medium and counted using a haemocytometer.

The cells were seeded in 100µl growth medium at $5x10^5$ nucleated cells per well in flat bottomed 96-well Nunclon™ tissue culture plates and incubated at 37°C, 5% CO_2 for 48h or 72h. LPS, (Sigma Chemical Co.) at 50µg/mL and conA (ICN Biochemicals, USA) at 10µg/mL, were added as mitogens at time zero. All experiments were performed in triplicate. Immediately prior to harvest, supernatants were collected for ELISA analysis and the cells were incubated for 2hrs in pre-warmed GM containing methyl-[^{3}H] Thymidine (TRK686, specific activity 80Ci/mmol, Amersham Biosciences) at a final concentration of 1µCi/mL. Cells were harvested using a Filtermate196, Packard harvester and counted using a Packard TopCount.NXT™ microplate scintillation and luminescence counter.

To determine the effect of LIF on Con A stimulation, BALB/c *axot*$^{+/+}$ splenic and thymic cells were incubated in the presence of Con A (2µg/mL or 10µg/mL) together with 500pg/mL or 1000pg/mL rmLIF (Santa Cruz Biotechnology, SC-4378). Mitogensis was measured as described above. Controls included GM only, conA only, and LIF only, at the respective concentrations.

ELISA

ELISA's were performed on the 48h culture supernatants, in 96-well Falcon® plates using the DuoSet® ELISAS for IFNγ (DY485), IL2 (DY402), IL4 (DY404), IL10 (DY417) and Quantikine®M Immunoassay for LIF (MLF00),

from R&D Systems. The standard curves were established by processing the optical density data using Microsoft Excel software and cytokine concentrations were determined using the standard curves.

Flow cytometry

The splenic and thymic cell suspensions were RBC depleted and washed in FACS staining solution (0.2% BSA and 0.1% sodium azide in 1xPBS) prior to being mixed with the various monoclonal antibodies detailed below, these being either directly or indirectly conjugated with Phycoerythrin (PE) or Fluorescein isothiocyanate (FITC). PE-rat anti-mouse CD19 (557399), PE-hamster anti-mouse TCRα chain (553172) and rat anti-mouse dendritic cell clone 33D1 (551776) were from Pharmingen. Rat anti-mouse CD205-FITC (MCA949F), mouse anti-rat IgG2a heavy chain-FITC (MCA278F) and mouse anti-rat IgG2b chain-FITC were from Serotec Ltd. while rabbit anti-mouse CD25 (IL2Rα) and goat anti-rabbit IgG (H&L)-PE (4050-89) were from Santa Cruz Biotechnology and Southern Biotechnology Associates respectively. Anti CD4 (YTS177.9.6) and anti CD8 (YTS 105.18.10) were a gift from Professor Stephen Cobbold, University of Oxford. Analyses were performed on a Becton Dickinson FACSCalibur instrument equipped with CellQuest software.

ACKNOWLEDGEMENTS

We are grateful to R. B. Heap for critical review of the manuscript and to S. Thiru for histological assessments. This work was supported by The Dunhill Medical Research Trust (S.M.M.), The Henry Smith Charity (H.L.T.), NIH: HD29471 (G.E.L.); GM077507-18 and MH11560 (M.A.H.). G.E.L is an Established Investigator of the American Heart Association.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Figure Legends

Figure 1.

DNA synthesis and cytokine release by splenocytes from *axot*$^{+/+}$, *axot*$^{+/-}$, and *axot*$^{-/-}$littermates

(**a**) H^3-thymidine labelling of spleen cells stimulated for 48h (upper panels) or 72h (lower panels) with conA (left-hand panels) or LPS (right-hand panels). DNA synthesis and standard deviation are shown after subtraction of the respective background controls for each genotype. Background controls were all less than 300cpm. (**b**) levels of IL2 and IL10 in supernatants at 48h after stimulation with either conA (upper panels) or LPS (lower panels). INFγ and IL4 were also

measured: INFγ was present in the conA culture supernatants only, the concentrations being 538pg/ml, 1410pg/ml, and 909 pg/ml respectively for *axot*$^{+/+}$, *axot*$^{+/-}$, and *axot*$^{-/-}$ cultures. IL4 was also only found in the conA supernatants and was 121pg/ml, 263 pg/ml, and 92 pg/ml respectively for *axot*$^{+/+}$, *axot*$^{+/-}$, and *axot*$^{-/-}$ cultures. The regression analyses for goodness of fit of each ELISA were as follows, IL2, $R^2 = 0.946$; IL4, $R^2 = 0.925$; IL10, $R^2 = 0.939$; and INFγ, $R^2 = 0.937$.

Figure 2.

Effect of axotrophin on LIF release.

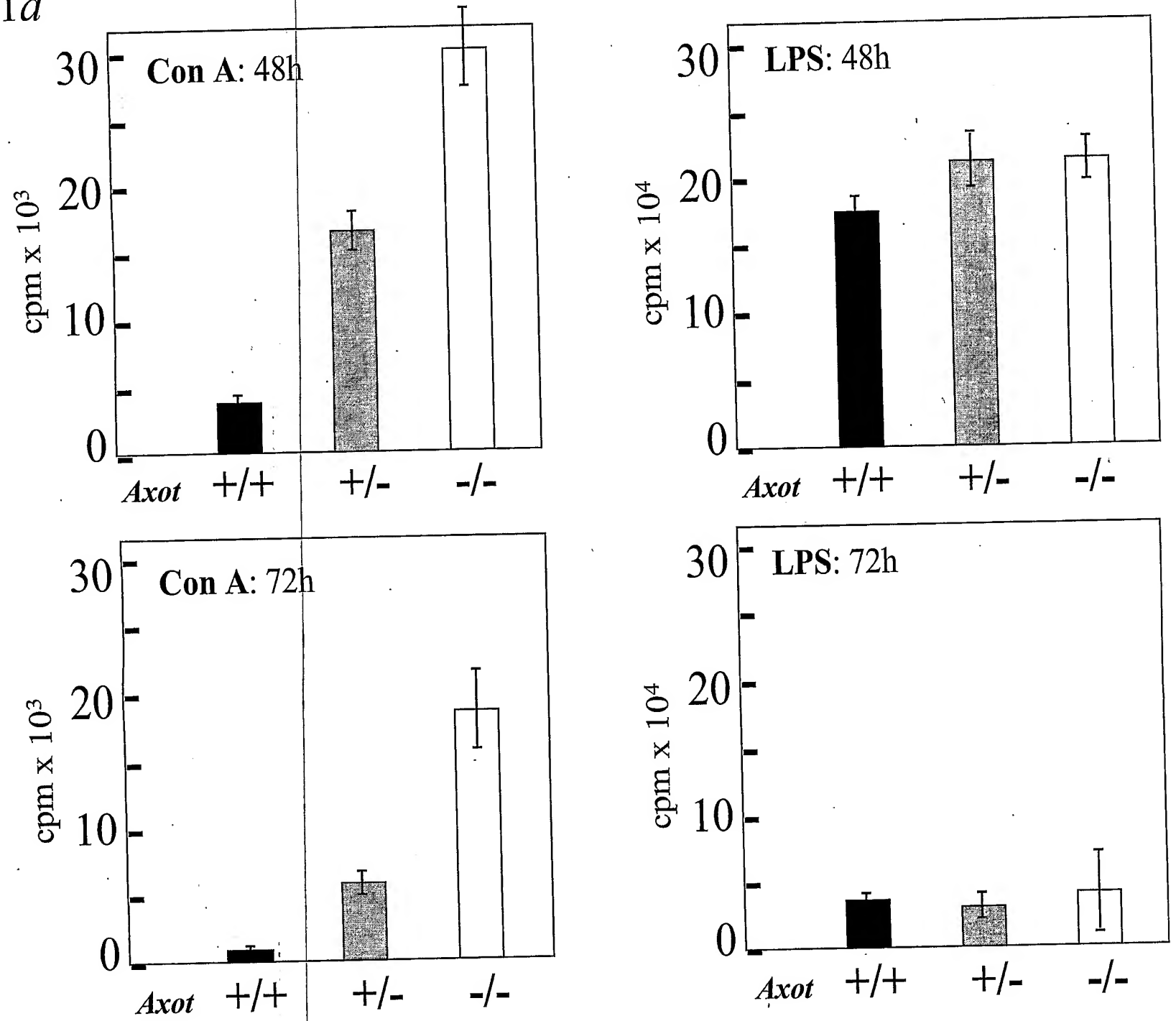
LIF release from spleen cells of *axot*$^{+/+}$, *axot*$^{+/-}$, and *axot*$^{-/-}$ littermates after 48h conA (left panel) or 48h LPS (right panel) stimulation. The regression analyses for goodness of fit was $R^2 = 0.999$.

Figure 3.

Phenotypic profile of spleen and thymus from *axot*$^{+/+}$ and *axot*$^{-/-}$ mouse littermates.

Whole populations of spleen and thymic cells were prepared, stained and analysed as described in Materials and Methods. The FACs data is presented in histogram format with the cut-off for negative staining indicated by the vertical line through each data set of CD4, CD8, CD3, CD19, DC33d1, and CD25 staining. The mouse *axot*$^{+/+}$ and *axot*$^{-/-}$ genotypes are as indicated above each panel. *Axot*$^{+/-}$ splenocytes and thymocytes were also analysed and gave the same results as those shown. CD205 staining was negative throughout (results not shown).

Fig 1*a*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cpm x 10³
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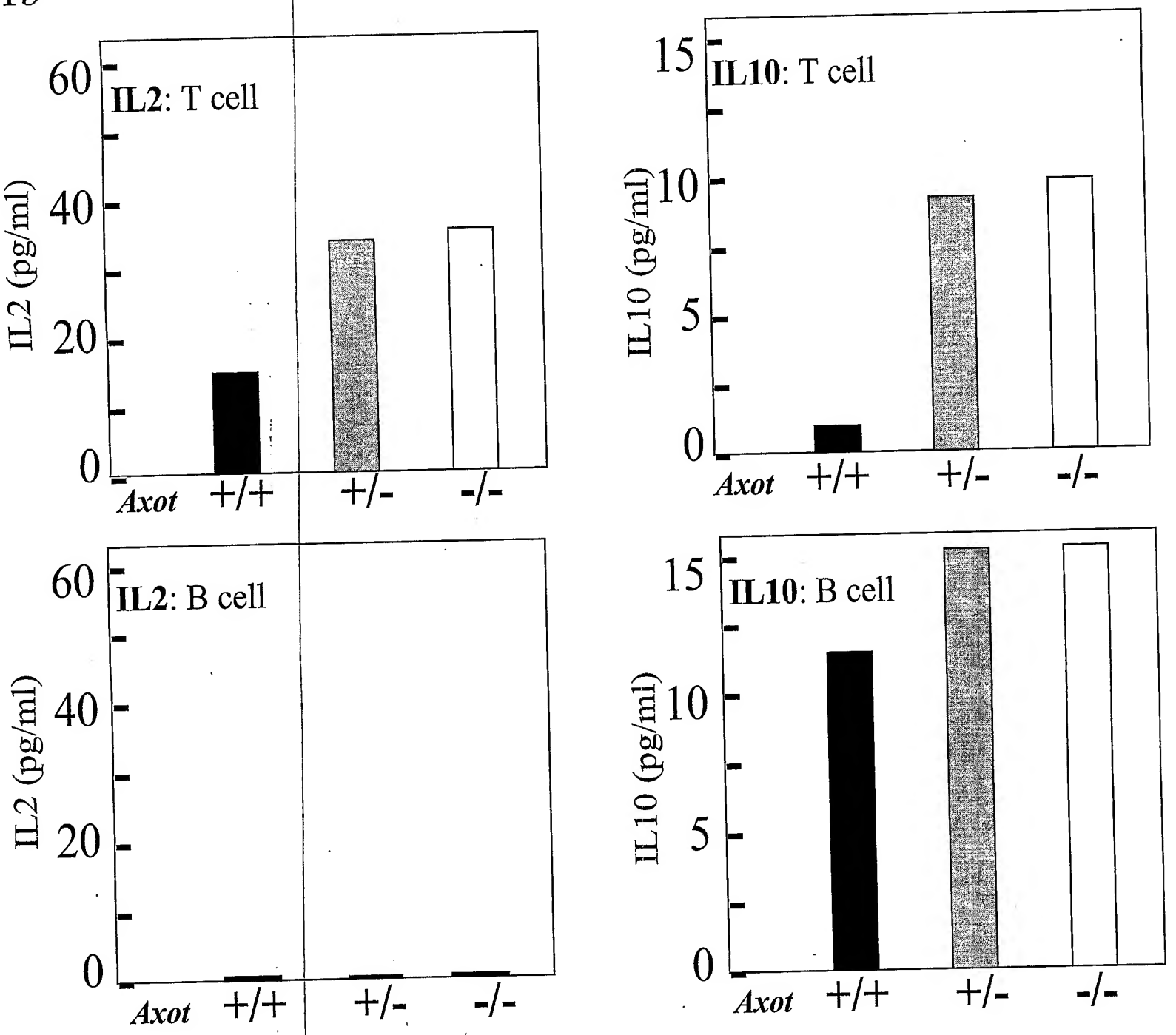
Con A: 72h
LPS: 72h

Fig. 1*b*


IL2: T cell
IL2 (pg/ml)
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IL10: T cell
IL10 (pg/ml)
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IL2: B cell
IL10: B cell

Fig. 2

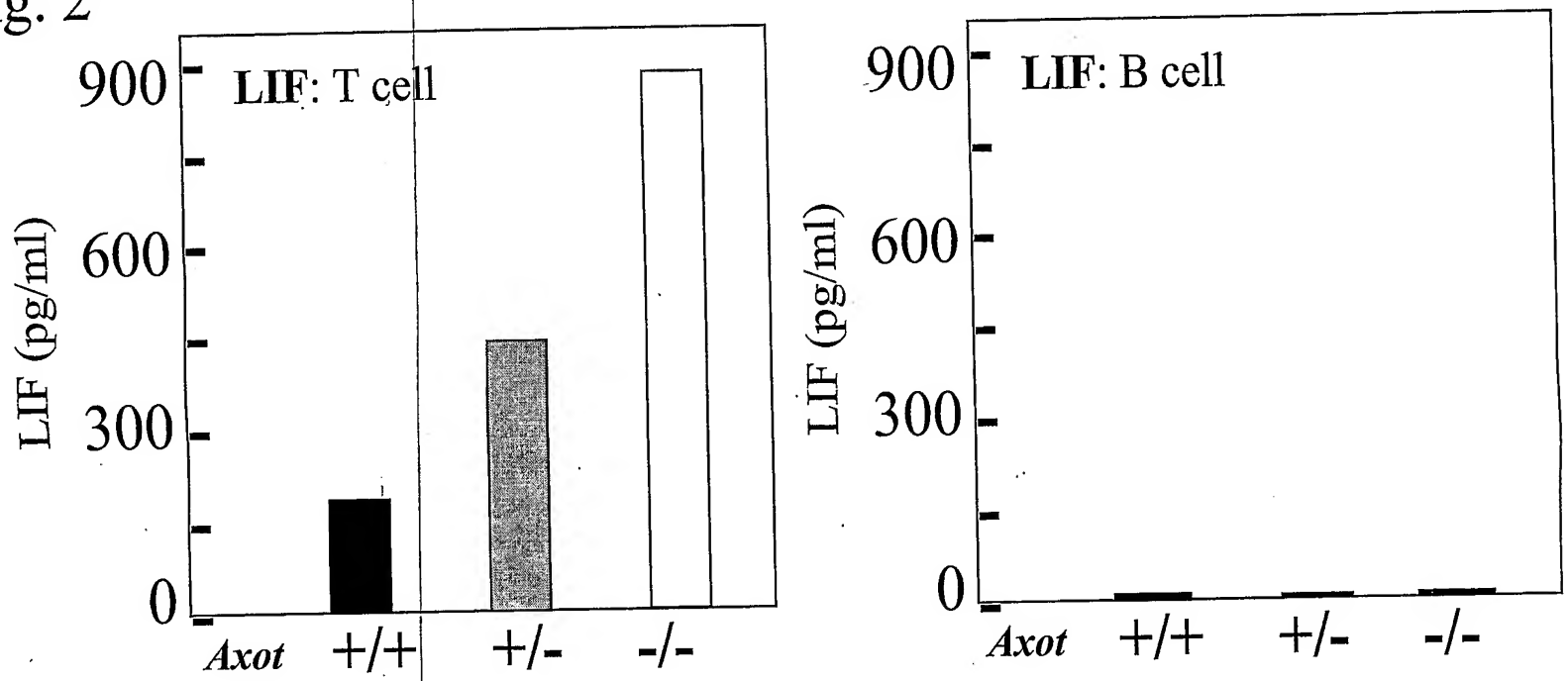
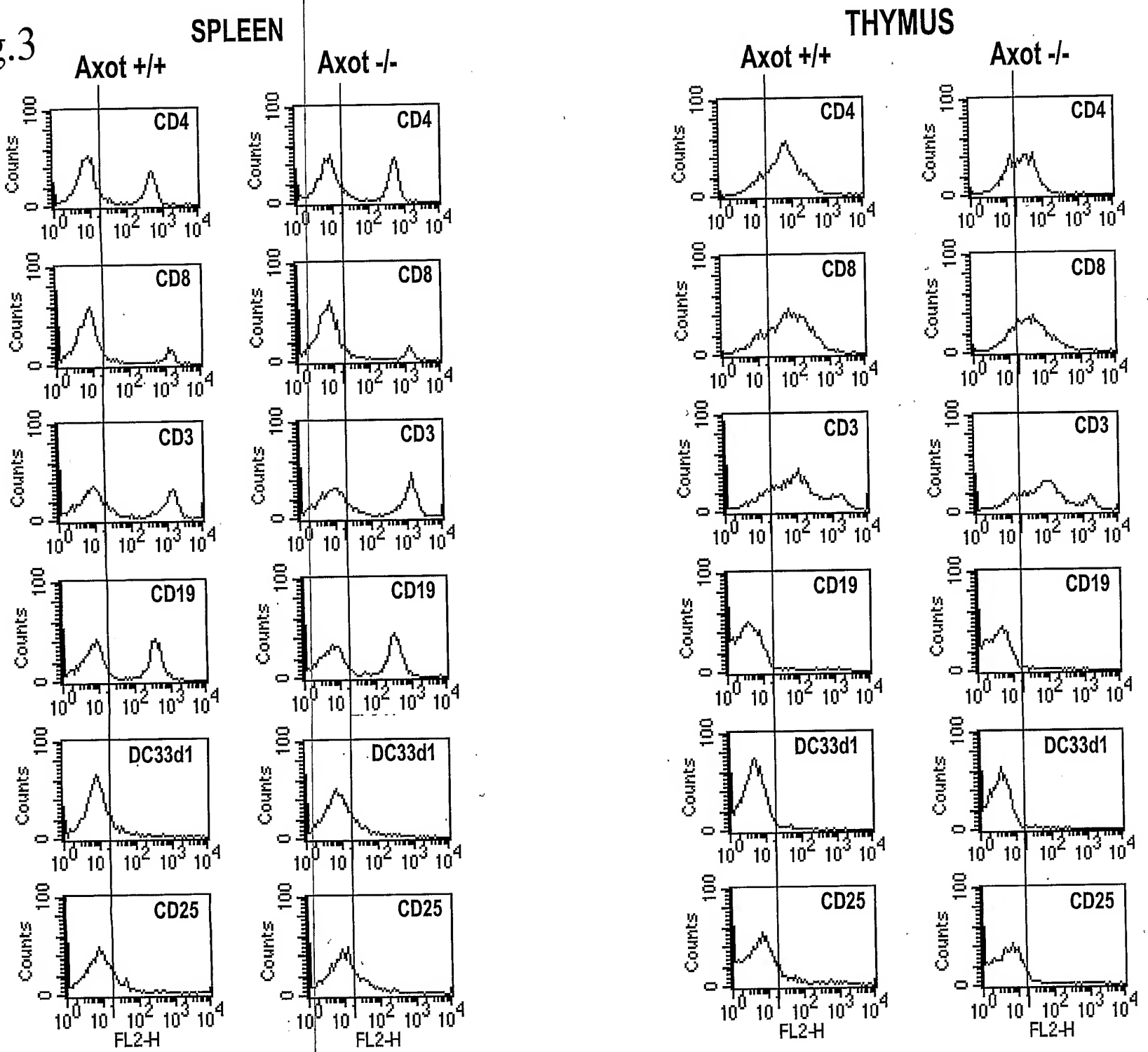

LIF: T cell
LIF (pg/ml)
900
600
300
0
Axot
+/+
+/-
-/-
LIF: B cell
LIF (pg/ml)
900
600
300
0
Axot
+/+
+/-
-/-

Fig.3


SPLEEN
THYMUS
Axot +/+
Axot -/-
CD4
CD8
CD3
CD19
DC33d1
CD25
Counts
FL2-H

p = 22

Leukaemia inhibitory factor, c-Kit and STAT3 correlate with regulatory tolerance: evidence from an ex vivo model in mice.

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Abstract. In an infectious tolerance model, spleen cells show immune regulatory properties and are able to impose donor-specific tolerance *in vivo*. Using spleen cells from either *BALB/c-tolerant*CBA, or *BALB/c-rejected*CBA mice, kinetic analyses of cytokines and transcription factors revealed that the most marked characteristic of the primed allo-aggressive response to donor was rapid IFNγ release, reaching 8000pg/ml @ 120h. The most marked characteristics of primed tolerance were high expression of STAT3 and c-kit, and release of LIF. Responses to third party were similar, indicating donor-specific responses in tolerance. The correlation between LIF and immune regulation was probed further using H-Y specific CD4+ clones: LIF exceeded 400pg/ml in Treg cultures, in marked contrast to low levels of LIF found in TH1 and TH2 clones of identical specificity. In conclusion, our data imply a role for SCF/c-kit, LIF/LIF-R, and STAT3 in transplantation tolerance and suggest a link between regulation of immunity and developmental control.

Key Words signal transduction; transplantation; tolerance

1. Introduction

Antigenic stimulation of naive T lymphocytes may induce T cell maturation along either Th1-type, or Th2-type, pathways. Here, signal transducers and activators of transcription (STATs) qualify differentiation towards either cellular immunity (STAT4; Th1) or humoral immunity (STAT6; Th2). Phenotypic cross-regulation occurs, as described in recent reviews [1-5] and the Th1 phenotype is characterised by IFN-γ release and activation of the Th1-specific transcription factor, T-bet. IFN-γ represses interleukin 4 (IL-4) cytokine release, thus blocking Th2 development. IL-4 characterises the Th2 phenotype and GATA3 is the Th2 specific transcription factor that transactivates the gene encoding IL-4, whilst GATA3 itself is induced by IL-4 receptor signalling, so setting up an autocrine loop for stabilisation of the Th2 phenotype. A third option for phenotypic differentiation is regulatory (Treg). The Treg phenotype has the power to suppress the activity of both Th1-type and Th2-type immune responses and Treg cells play a critical role both in self-tolerance and in acquired tolerance to transplanted allo-antigen [6-14]. The regulatory mechanisms responsible for self tolerance and transplantation tolerance appear to differ. In self tolerance, regulatory T cells arise centrally within the thymus; are positive for surface expression of both CD4 and CD25 (IL2 receptor alpha chain); and show constitutive expression of CTLA-4. Such cells are anergic and impose anergy within the immediate environment in a non-specific manner *via* release of IL-10 and TGF-β [14], also reported to occur in transplantation tolerance for IL10 [15]. Treg-mediated immuno-suppression involves signalling through the glucocorticoid-induced tumor necrosis factor receptor family related gene product (GITR) that is thought to inhibit co-stimulatory pathways [16]. The thymic development of CD4+ CD25+ Treg has been recently demonstrated to require scurfin, a forkhead transcriptional repressor encoded by the Foxp3 gene [17, 18, 19].

In murine transplantation tolerance, regulatory $CD4^+$ T cells can be generated in the periphery of the recipient following a brief period of therapy, such as blockade of CD4/CD8 co-receptor function. Both euthymic, and athymic, recipients become permanently tolerant by such brief treatment, and experiments have shown (i) that peripheral allo-antigen-responsive CD4+ T cells can be induced to mature towards dominant regulatory cells, and (ii) that naïve allo-reactive CD4+ T cells emerging from the thymus acquire the tolerant phenotype, even after therapeutic antibody has been cleared [12]. The resultant transplantation tolerance is allo-specific for the donor and is self sustaining, imposing itself on new cohorts of lymphocytes as they arise [12, 20]. The dominance of Treg cells extends to suppression of primed allo-aggressive spleen cells transferred into the tolerant mouse [20]. No allo-antibody is detectable in these tolerant mice, implying co-suppression of B-cell allo-responsiveness [21] and there is very good vascular preservation within the graft at 1 year [22]. Furthermore, spleen cells isolated from tolerant mice carry the property of transplantation tolerance and are sufficient to impose specific tolerance when transferred into untreated, fully immuno-competent recipients [12]. Such adoptive transfer of allo-specific tolerance is successful over serial generations of recipients [12], so demonstrating "infectious tolerance" and amplification of the tolerant phenotype. Both CD4+ CD25+, and CD4+ CD25-, cells can mediate transplantation tolerance, and each is associated with distinct gene expression profiles [13]. Continued presence of allo-antigen is necessary to retain the tolerant state and removal of allo-antigen results in loss of tolerance within seven days [12]. Thus, maintenance of dominant transplantation tolerance appears to have two absolute requirements: allo-specific CD4+ Treg cells, and continuous presence of allo-antigen .

The Th1- and Th2-type immune responses are open to molecular analysis *via* the conventional mixed lymphocyte response (MLR) using either mixed or purified cell populations. In contrast, the intracellular signal transduction pathways associated with regulatory tolerance have yet to be characterised. To identify pivotal molecular events that distinguish tolerance from rejection we have undertaken a detailed analysis of spleen cells obtained from tolerant animals and known to be capable of adoptively transferring specific tolerance *in vivo*. This provided us with a source of cells that carry the property of specific allo-tolerance for molecular analysis.

Freshly isolated spleen cells from CBA mice that were tolerant to a BALB/c heart graft (herein termed *BALB/c-tolerant*CBA) were challenged with irradiated BALB/c spleen cells as a source of donor antigen. Comparisons were made with *BALB/c-rejected*CBA spleen cells similarly challenged with BALB/c. STAT 3 was found to be specifically up-regulated during the early tolerant response and this was coincident with specific up-regulation of c-kit, the receptor for stem cell factor (SCF). The tolerant response was also associated with selective release of leukaemia inhibitory factor (LIF), a factor known to alter differentiation pathways in stem cells [23].

Materials and Methods

Generation of BALB/c-primed CBA mice.

All mice used were male and were obtained from Harlan U.K.). CBA mice ($H2^k$) of 10 - 12 weeks of age received a fully mismatched, vascularised BALB/c ($H2^d$) heart graft to the neck, using the technique described by Chen [24]. Tolerance was generated by a 21 day course of alternate day therapy using blocking mAbs to CD4 and CD8 as previously described [12]. After 40d, levels of therapeutic antibody in the peripheral circulation become undetectable [25]. *BALB/c-tolerant*CBA spleen cells from tolerant recipients were isolated at least 100d after grafting for *ex vivo* analyses. For comparison, untreated CBA mice, 10 - 12 weeks of age, were grafted either with BALB/c tail skin which rejected by day 10, or with a BALB/c heart which rejected on day 7. The *BALB/c-rejected*CBA spleen cells were collected at 14d for *ex vivo* analyses. All procedures were carried out according to Home Office licence under the Animals (Scientific Procedures) Act 1986, UK.

Ex vivo model

The *ex vivo* model is described in detail elsewhere [26]. It uses whole spleen cell populations in order to mimic the *in vivo* state of transplantable tolerance [12]. There are important differences between the ex vivo model and a conventional MLR: the responder "tolerant" and "rejector" spleen populations are each derived from mice primed *in vivo* against donor antigen. Moreover, the cultures are amplified for responsiveness to donor, both due to *in vivo* priming, and thereafter by *ex vivo* boost with donor antigen, when some 18-fold amplification of donor responsiveness may occur. No exogenous cytokines are added. Thus, any differences in responses against donor are due to inherent differences in the donated spleen.

Responder spleen cells were obtained from either *BALB/c-tolerant*CBA, or *BALB/c-rejected*CBA, mice, and each series was stimulated by irradiated spleen cells from (i) BALB/c (donor antigen), (ii) CBA (self antigen), or (iii) C57Bl10 ($H\text{-}2^b$; third party antigen). Each culture flask contained $4x10^7$ responder cells and $6x10^7$ irradiated (20Gy) stimulators in a total of 10ml growth medium (GM, Iscoves + 10% FCS + 100 U/ml each of penicillin and streptomycin). After 120h, 500 microL of GM containing a further $6x10^7$ irradiated stimulator cells was added to

each flask to re-boost the immune response. Replicate flasks were removed at 0h, 16h, 24h, 48h, 120h (immediately after re-boosting), 121h, 123h, 126h and 144h. The culture supernatant at each time point was spun free of cells and stored at -80°C.

Cell Lysis.

Cells were collected onto ice, with any adherent cells being included following brief treatment with 0.25% trypsin. After washing in ice cold 0.1% BSA/PBS, the cells were separated into cytoplasmic and nuclear fractions as previously described [26]. All fractions were stored at -80°C.

ELISA

Culture supernatant was analysed for the presence of IFN-γ, IL-2, IL-4, IL-10, IL-12, IL-13, SCF and LIF. ELISA assays used DuoSet kits from R&D Systems as follows: IFN-γ (DY485), IL-2 (DY402), IL-4 (DY404), IL-10 (DY417), IL-12p70 (DY419), IL-13 (DY413), TGF-β (DY240) and SCF (DY455). The R&D Systems Quantikine mouse LIF ELISA (MLF00) was used to measure LIF.

Western blot

Antibody probes for STAT 1 (sc346), STAT3 (sc7197), STAT5b (sc836), STAT6 (sc621), c-kit (sc5535), SCF (sc1303) and IL2Rα (sc666) were obtained from Santa Cruz Biotechnology USA. Anti-STAT4 was obtained from R&D Systems (PAF-ST4). Anti-actin (MAB1501) was from Chemicon International. The Pharmacia Phast system was used for SDS-PAGE and Western blotting of lysates. After protein transfer, membranes were probed for target protein using standard proceedures, followed by chemiluminesence detection by ECL-Plus (Amersham International). The membranes were then quenched by overnight incubation in PBS-azide 0.1% Tween plus 5% milk at +4°C and reprobed for actin. Each signal was quantified by image analysis using GelDoc imaging and software. To standardise direct comparisons of cytoplasmic protein levels, the O.D. of each protein was measured and compared to the O.D of the actin signal in the same sample lane. The protein to actin ratio at time zero was assigned a value of 100% and changes in this ratio were plotted on linear scale.

Experimental reproducibility

To ensure direct comparablity between each experimental time point, and between responses to different stimulators, all cultures within a given responder series utilised a common stock of responders (ie primed tolerant, or primed rejection). The results for IL2Rα levels (Figure 1) illustrate the high reproducibility between two series recorded at serial time points over a period of 144h. The intra-experimental reproducibility was also high, with a maximum of 10% variation for six repeats of actin measurements by western blot. A total of 6 *ex vivo* experiments gave constant relative relationships when comparing tolerance with rejection.

2. RESULTS

(i) IL2 activity is similar in both tolerant and rejection responses

IL2 release in response to donor antigen, and IL2-Rα expression, was very similar in responders from tolerant, and rejector, spleens (Table 1, and Figure 1). The kinetics of IL2 accumulation, and its consumption at later time points, was equivalent. For the IL2Rα, freshly isolated spleen cells showed significant background expression followed by

progressive signal loss. Upon re-boosting at 120h, the 55kDa band was recovered, due in part to the background IL2Rα added in by the freshly added cells. Again, there was progressive loss of the 55kDa band, concomitant with progressive increase in a 75kDa product that was first detected at 120h and thus corresponded to developing responses (ie not background). Figure 1 shows the levels of IL2 measured in the supernatants of the corresponding cell samples. We interpret the 75kDa band as representing stable IL2-IL2Rα complexes.

(ii) Rapid release of high INFγ levels characterises the rejection response.

Table 1 shows rapid accumulation of INFγ in the rejecting arm of the experiment, reaching 720pg/ml at 16h. This extrapolates back to INFγ release first becoming detectable at 7h. In marked contrast, the *BALB/c-tolerant* CBA spleen cells showed a delayed and low level INFγ response. At 120h the level of INFγ was over 6 times greater in rejection (8,551 pg/ml) compared to tolerance (1,378 pg/ml). Others have reported normal Th1 cytokine production by unfractionated peripheral lymphocytes from tolerant mice [15]. It is possible that demonstration of the striking suppression of INFγ in tolerance found here requires fully matched comparisons (tolerance *versus* rejection) and serial measurements.

(iii) Evidence of *in vivo* priming to donor.

By comparing responses to donor with responses to third party allo-antigen, we were able to demonstrate in vivo priming to donor. Figure 2, right-hand panel shows that the INFγ response to third party antigen was greatly reduced and delayed compared to the INFγ response against donor (left-hand panel). Thus we deduce that CBA mice that had rejected a BALB/c skin or heart graft contained primed Th1-type donor-responsive spleen cells. Previous *in vivo* experiments have established that spleens from tolerant CBA mice contain donor-primed CD4+ T cells [12].

(iv) STAT3 and c-kit showed enhanced levels in rejection.

There was a remarkable similarity in the induction kinetics of STAT4 when comparing tolerance with rejection (Figure 3a). Both showed an initial four to five-fold increase, and both returned to baseline levels by 120h. Upon secondary stimulation, STAT4 levels remained constant (120h, 121h, 123h, and 126h) and then showed a delayed, seven fold increase in both culture phenotypes (144h).

The behaviour of STAT3 (Figure 3b) was in marked contrast to STAT4. Challenge with donor caused a specific decrease in STAT3 in the *BALB/c-rejected*CBA spleen cell cultures, whilst STAT3 levels more than doubled in the *BALB/c-tolerant*CBA spleen cell cultures. This increase in STAT3 was maintained and showed yet further increment in response to secondary stimulation. In contrast, STAT3 in the rejection response showed recovery of baseline expression at 120h followed by a rapid decrease upon re-boosting. Thus, priming to donor resulted in divergent STAT3 behaviour, qualified by response phenotype.

Of the STATs, the single most notable feature of the *BALB/c-tolerant*CBA cultures was a sustained induction of STAT3. No striking differences were seen in STAT 1 levels in any of the samples. Levels of STAT5 was very low and appeared similar in both tolerance and rejection. STAT6 was induced, with higher levels in rejection compared to tolerance, and returned to baseline levels by 120h (Figure 3c). Thus, the responses of STATs 4, 5, and 6 were all transient in both tolerance and rejection, as also was STAT3 in rejection. The transient increases of STAT6 in the

rejecting cultures at 120h – 126h contrasted with neglible changes in STAT6 in tolerance. Since STAT6 is a feature of Th2 regulation, this result indicates that the *ex vivo* tolerant response is not of a Th2-type.

Since STAT3 may transduce signals resultant from SCF / c-kit interactions, we also probed the samples for both SCF and c-kit by Western blot, and assayed for soluble SCF in the culture supernatants. Neither soluble nor cell-associated SCF was detected. However, c-kit was seen and showed major kinetic changes relative to actin. At 16h and 48h, c-kit was significantly induced in both tolerance and rejection, with greater induction in tolerance (Figure 3d). The induced levels of c-kit were maintained and upon restimulation showed further induction, with tolerance again being the greater of the two. When the nuclear fractions were probed, full length c-kit protein was found in the nucleus at 48h (Figure 4). This was confirmed in a total of eight preparations from individual flasks at 48h. No actin was detected in the 48h nuclear fraction, excluding the possibility of cytoplasmic contamination.

(v) LIF release is increased in Tolerance

Having demonstrated a relationship between c-kit and tolerance, we next asked in LIF shows differential expression in tolerance. Supernatants from tolerant cultures contained ten times more LIF than the rejecting culture supernatants at 120h (Table 3a). This apparent correlation between LIF and tolerance was pursued by comparing culture supernatants from Th1, Th2, and Treg, CD4+ T cell clones that are mono-specific for the H-Y antigen [27]. Using 7d supernatant, or 18h supernatants from anti-CD3 + anti-CD28 treated clones, we found that only the Treg clone showed high LIF release (Table 3b). SCFs was not detected in any of these clone supernatants, but IFNγ and IL-10 showed the expected polarisation towards Th1, and Th2/Treg, respectively (Table 3b). Since (i) both the Th1 and Th2 clones can rapidly reject male skin grafts *in vivo*, and (ii) the Treg clone not only fails to reject male skin grafts, but suppresses the ability of the Th1 and Th2 clones to reject male skin grafts [27], these results support the concept of LIF having a role in regulatory transplant tolerance.

DISCUSSION

The specific regulation of allo-tolerance *in vivo* occurs in a complex micro-environment and involves co-operation between different cell types. Using an un-manipulated *ex vivo* model aimed to mimic this *in vivo* situation, we have identified novel candidates for operation of pivotal signalling pathways in regulatory transplantation tolerance, namely c-kit, LIF, and STAT3.

The potential role for STAT3 in immune regulation was deduced from its divergent behaviour between tolerance and rejection responses, with specific induction in tolerance. The STATs are a family of seven transcription factors, each with unique, non-redundant functions in receptor-mediated signal transduction of the cytokine receptor superfamily, recently reviewed by Ihle [28]. STAT-activity is in turn negatively regulated by phosphatases and suppressors of cytokine signalling (SOCS) to recover normal responsiveness to cytokine stimulation [29, 30]. STAT 3 is relatively versatile and has been found to be activited by c-kit [31], in addition to the IL6 family cytokines and growth factors. STAT3 knock out is embryonic lethal but tissue-specific targetting studies have identified STAT3 as having cell lineage-specific activity, where T cell development appears to be STAT3 independent. Loss of STAT3 in macrophages results in their constitutive activation, supporting a role for STAT3 in the anti-inflammatory responses induced by IL10 [31]. This, together with our current findings, lead us to anticipate that STAT3 may operate at multiple levels during regulatory tolerance. LIF release was found to be associated with tolerance, and LIF/LIF-R

mediated signalling activates STAT3 [32]. C-kit showed enhanced induction in tolerance that was in parallel with the induction of STAT3. Thus SCF/c-kit and LIF/LIFR, through STAT3, may interact, or act co-operatively, within common pathways of immune regulation.

SCF is a pleiotrophic growth factor and in these experiments SCF activity was inferred by the dynamic behaviour of c-kit levels. However, the SCF ligand appears to be rapidly consumed since we were unable to detect either membrane bound, or soluble, SCF in any of the samples. C-kit was detected and showed enhanced induction in tolerance. Splenic T cells from CBA mice include a small sub-population of c-kit+ TCR αβ cells [33], and c-kit plays a key role in T cell lineage development and maintenance of lymphopoiesis [34, 35]. A type III receptor tyrosine kinase, c-kit activates a variety of signalling pathways, including those mediated by STAT3. SCF-ligated c-kit becomes internalised and rapidly degraded by the ubiquitin pathway [36]. The observed nuclear translocation of full length c-kit is a novel finding which may indicate direct transcriptional competence. Of relevance is the recent report that the EGF receptor undergoes nuclear localisation [37], lending credence to the concept of dual activity wherein certain cell surface receptors also function as transcription factors [38]. If c-kit is found to be competent as a transcription factor, then genes accessible for trans-activation by c-kit in the tolerant phenotype are likely to differ from those of the rejection phenotype due to epigenetic changes influencing gene profiles accessible for transcription. Future analyses will compare gene expression profiles at the 48h time point to look for such putative c-kit target genes.

LIF is also a pleiotrophic growth factor and LIF signalling is associated with specific activation of STAT3 [32]. LIF belongs to the IL6 family of cytokines that control differentiated cell functions of those cells which express the appropriate receptor proteins. The expression profile of these receptors is established during differentiation and is subject to regulation by histone deacetylation. Reactivation of epigenetically silenced genes induces expression of both STAT3 and LIF receptor alpha (LIF-Rα) in spleen cells [39]. LIF is produced by a variety of cell types including CD4+ve Th2-type lymphocytes [40] and is a natural regulator of immune tolerance at the materno-fetal interface [40]. We found that release of LIF characterised *ex vivo* tolerance and we were able to identify Treg as a major source of LIF, this being significantly greater than cells of the Th2-type. It is notable that SAGE analyses of Treg, TH1, and TH2 clones failed to highlight LIF as being associated with tolerance, although cultures of Treg were found to be associated with over-expression of mast cell genes (the mast cells being part of the feeder cell population) [27]. Since both SCF and LIF are mast cell growth factors, our findings might predict an association between mast cell activity and regulatory tolerance and explain the previous findings from SAGE.

A link between regulatory tolerance and developmental signalling pathways is here extended to include both SCF and LIF, in addition to the Notch family of proteins discovered by others [9]. Our current working hypothesis is as follows: (i) that naïve T cells are open to various fate determination pathways, one of which leads to the regulatory tolerant phenotype; (ii) that this regulatory phenotype represents an early developmental stage of the activated niave T cell which is stabilised by LIF/LIF-R and SCF/c-kit activity; and (iii) that, once established, this phenotype is dominant over TH1 and TH2 phenotypes so preventing an aggressive immune response. We are currently investigating induced gene expression characterising the tolerant response and preliminary data suggets selective amplification of ELKL motif kinase (required for immune system homeostasis in the mouse [41]) and axotrophin (a ring-type zinc finger transcription factor involved in CNS development [42]) but shows no evidence of differential

expression of Foxp3. If confirmed, Foxp3 may be functionally linked to an ELKL motif kinase; alternatively, more that one pathway capable of imposing regulatory tolerance *in vivo* may exist.

The models we have used are proven as imposing a specific and dominant state of tolerance *in vivo*, a fact which enhances our long term aim of exploiting natural regulatory pathways for therapeutic manipulation of immunity, either *in vivo* or *ex vivo*. Here, there are direct implications for treatment of auto-immune disease, and for the potential clinical application of pluripotent cells, in addition to inducing graft-specific tolerance in transplant recipients. The work also identifies novel candidates for surrogate markers of immune tolerance. In conclusion, our *ex vivo* analyses have identified LIF, STAT3 and c-kit as being linked to the regulation of transplantation tolerance.

Acknowledgements

We are grateful to Professors Herman Waldmann and Stephen Cobbold of the Dunn School of Pathology, Oxford, for gifts of the YTS 105 and YTS 177 monoclonal antibodies, and to Professor Andrew Bradley for constructive comments on the manuscript. Su Metcalfe is a Dunhill Medical Research Fellow: this work was also supported by grants to SMM from the Stanley Thomas Johnson Foundation and from Fujusawa Pharmaceuticals.

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Figure Legends

Figure 1.

Western blot for IL2Rα comparing cytoplasmic fractions of 'tolerant" and "rejecting" spleen cell cultures after primary and secondary stimulation with donor (BALB/c) allo-antigen. The levels of IL2 in the corresponding culture supernatant (background subtracted) is shown beneath each lane: the background IL2 values found in CBA->CBA control cultures are given in Table 1. The arrows indicate first *ex vivo* challenge (0 hour) and secondary *ex vivo* challenge (120 hours) with irradiated BALB/c spleen cells.

Figure 2.

Comparative kinetics of INFγ release from "tolerant ", *versus* " rejecting", spleen cells during the first 48h of allo-stimulation with either donor (BALB/c, left-hand panel), or third party (C57Bl/60, right-hand panel), irradiated spleen cells.

Figure 3.

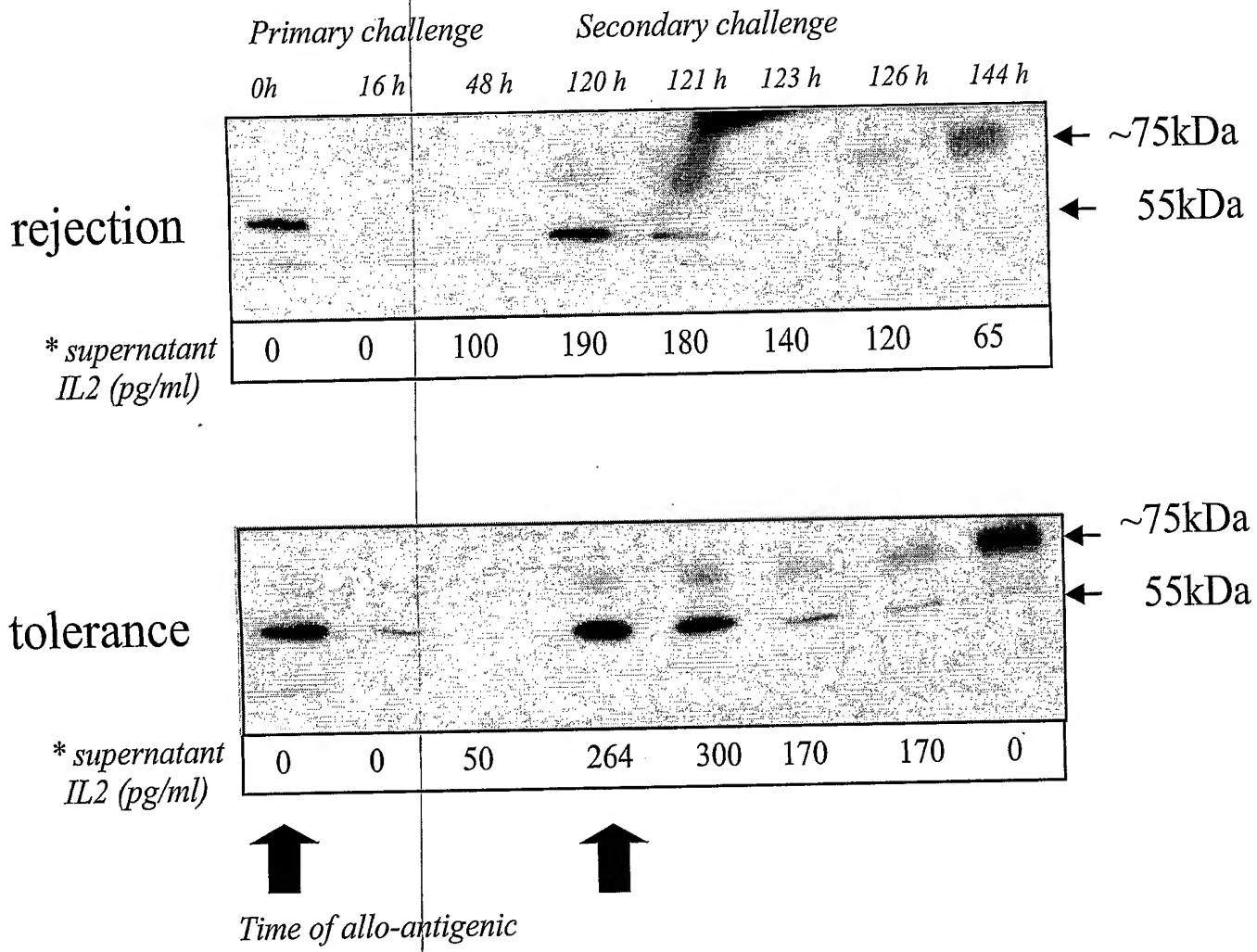
Levels of cytoplasmic STAT4, STAT3, STAT6 and c-kit in "tolerant" and "rejecting" spleen cell cultures. Primary Western blots for each target protein were re-probed for actin. The ratio between target protein and actin in each lane was obtained from the O.D. values: the zero hour time point was nomalised to 100%. To illustrate the derivation of the presented data, the raw data for the rejection series [STAT4 O.D : actin O.D] were as follows: [43,524 : 19,410] at 0h; [23,338 : 7,819] at 16h; [17,793 : 2,161] at 48h; [45,941 : 24,901] at 120h; [31,684 : 13,600] at 121h; [18,575 : 12,551] at 123h; [16,339 : 7,193] at 126h and [30,345 : 1,773] at 144h. The same sample stock was used for the four series of primary probes shown : Figure 3(a) STAT4; Figure 3(b) STAT3; Figure 3(c) STAT6; and Figure 3(d) c-kit.

Figure 4.

Nuclear translocation of c-kit . Cytoplasmic and nuclear fractions of "tolerant" and "rejecting" spleen cell cultures were Western blotted for c-Kit at time zero (0h) , 16h and 48h after primary challenge with irradiated BALB/c spleen cells. The blots were quenched and then reprobed for actin : the lowest panel show the actin signal in the "rejecting" samples. The level of nuclear actin at time zero was often relatively high, relating to high numbers of G0 cells prior to activation. By 48h the level of nuclear actin was negligible even after over-exposure (seen here), but the same sample lane contained significant levels of full length c-Kit protein.

Fig 1

IL2 receptor α : CD 25



* net after subtraction of IL2 in CBA/CBA control cultures

Fig 2

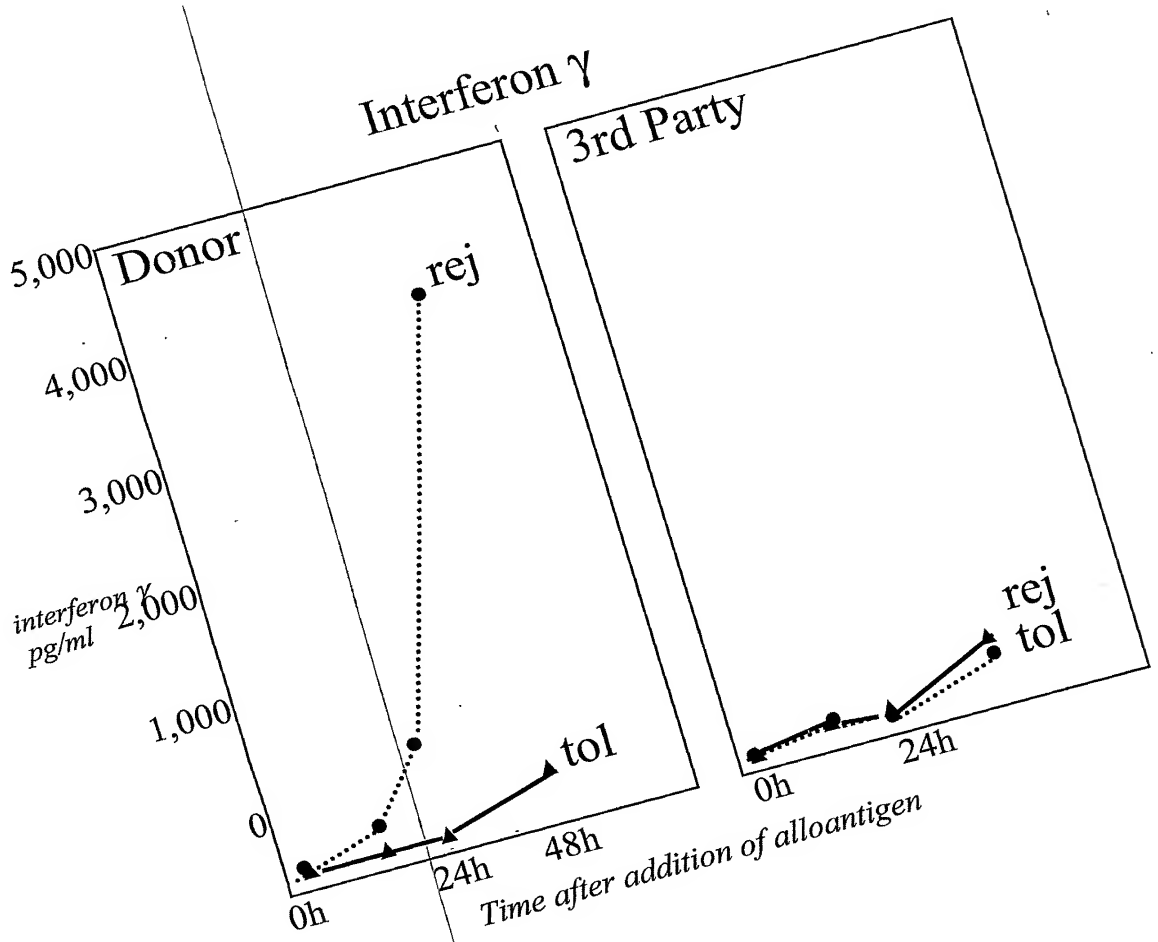

Interferon γ
Donor
3rd Party
rej
tol
rej
tol
5,000
4,000
3,000
2,000
1,000
0
interferon γ
pg/ml
0h
24h
48h
0h
24h
Time after addition of alloantigen

Fig 3

a.

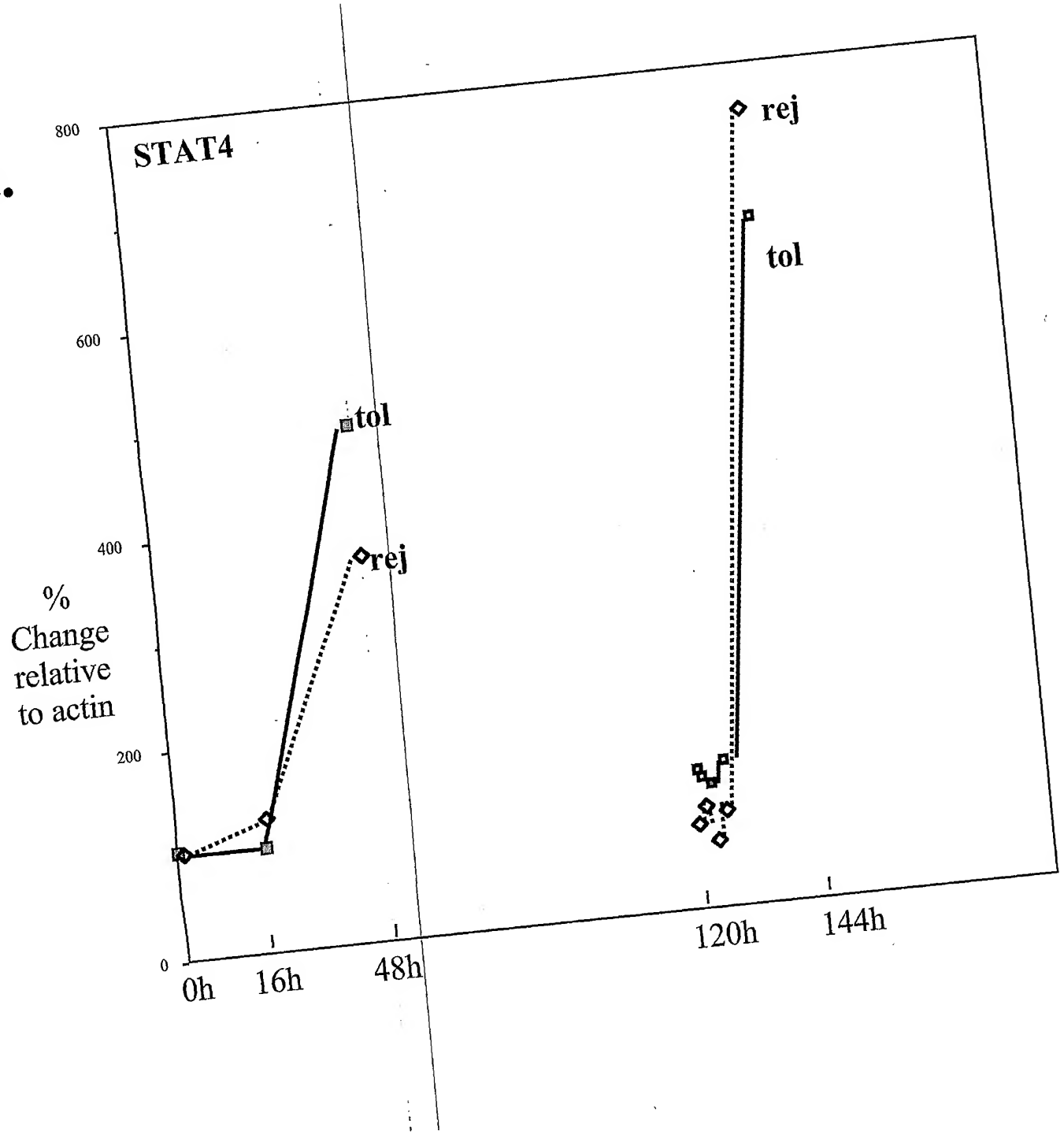

STAT4
800
600
400
200
0
% Change relative to actin
tol
rej
tol
rej
0h
16h
48h
120h
144h

Fig 3

b.

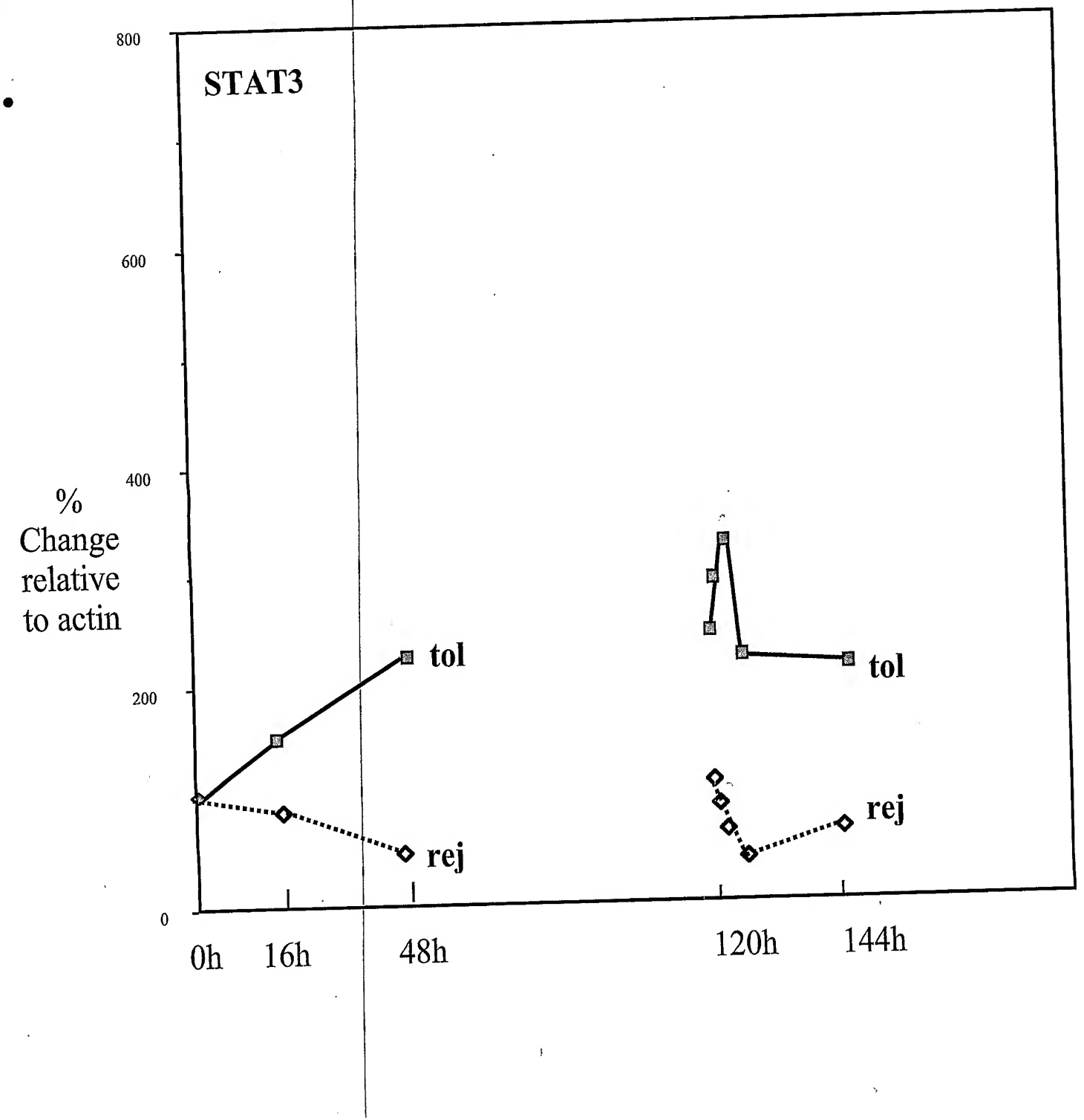

STAT3
800
600
400
200
0
% Change relative to actin
tol
rej
tol
rej
0h
16h
48h
120h
144h

Fig 3

c.

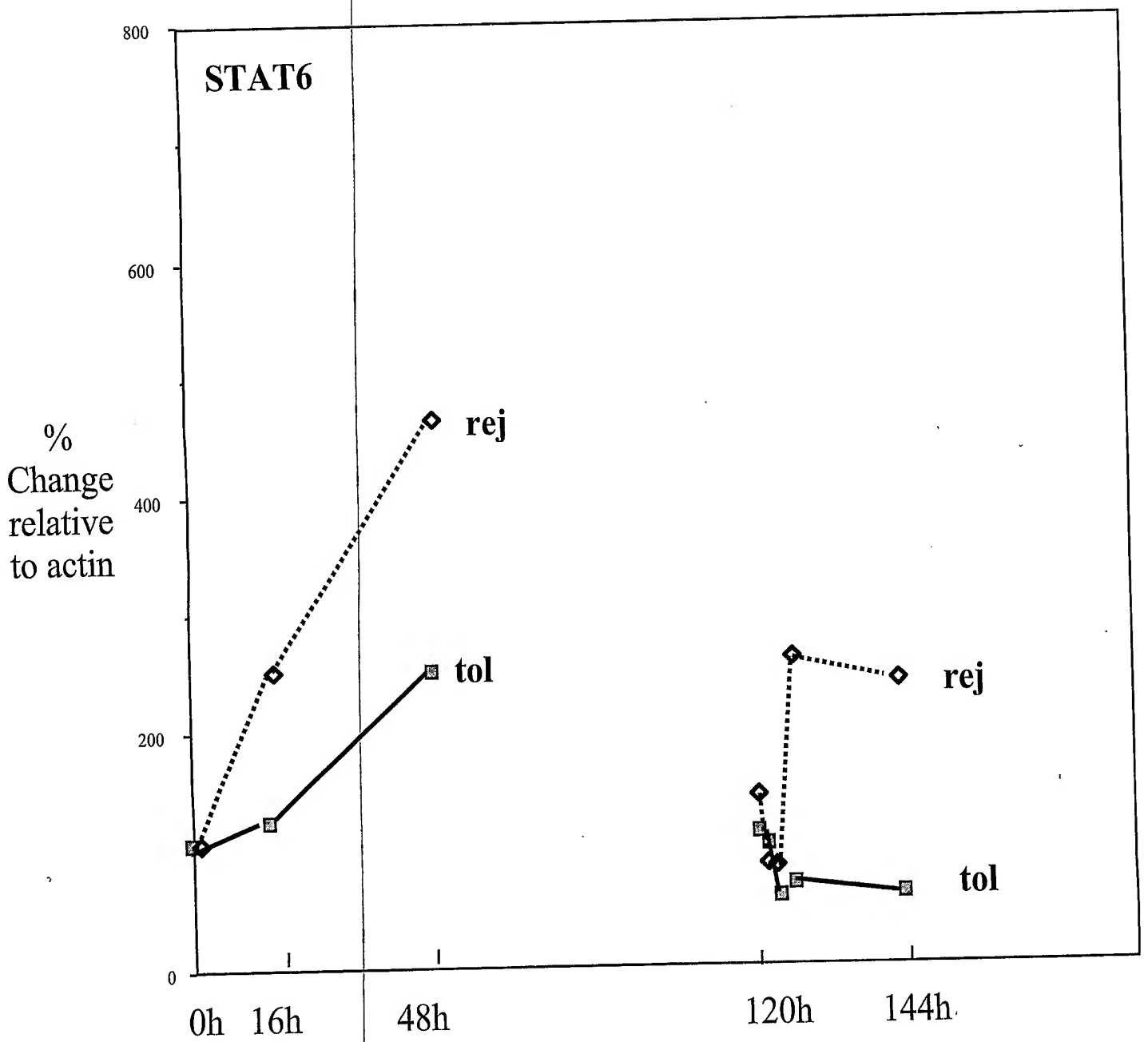

STAT6
800
600
400
200
0
% Change relative to actin
rej
tol
rej
tol
0h
16h
48h
120h
144h

Fig 3

d.

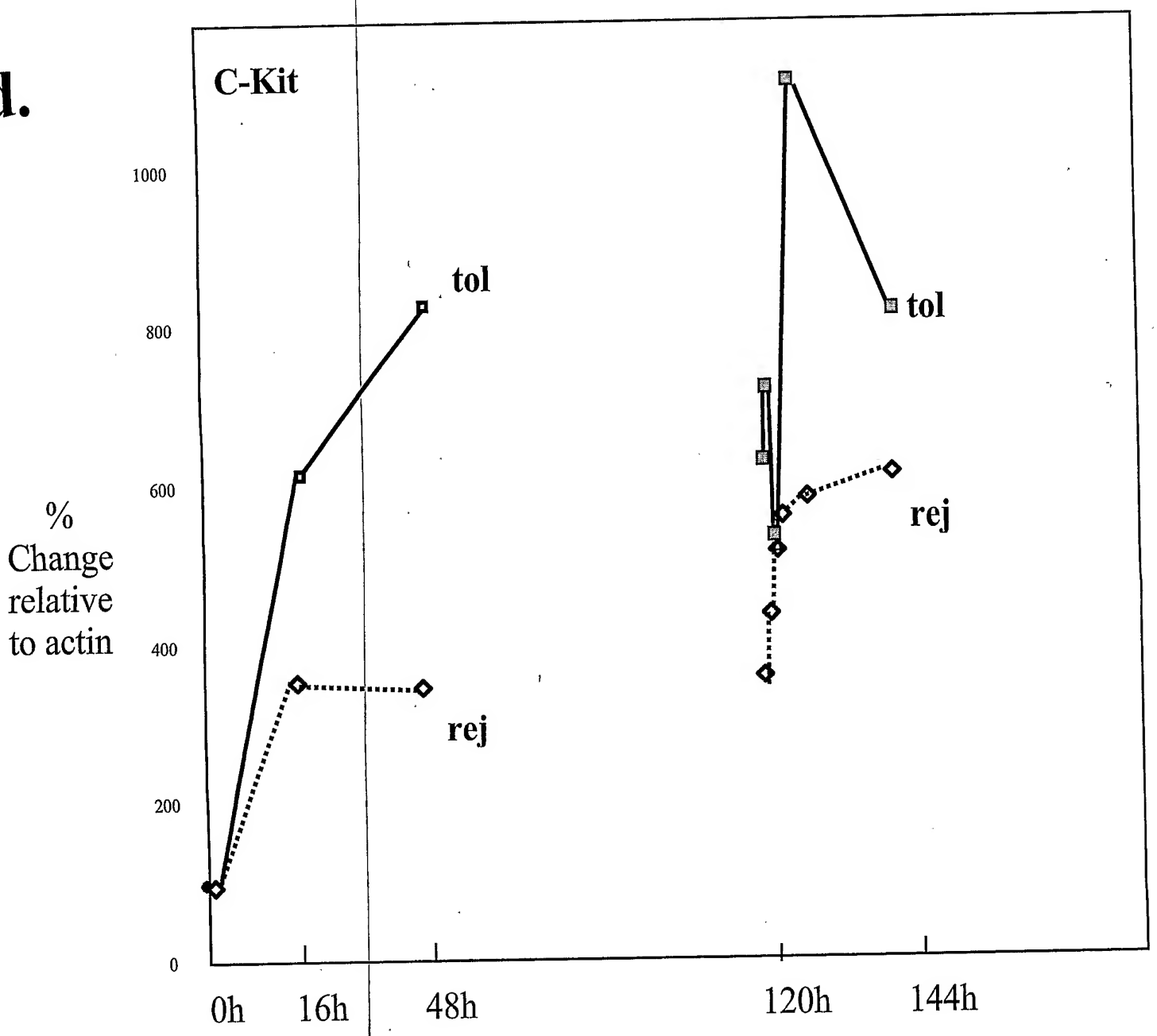

C-Kit
1000
800
600
400
200
0
% Change relative to actin
tol
rej
tol
rej
0h
16h
48h
120h
144h

Fig 4

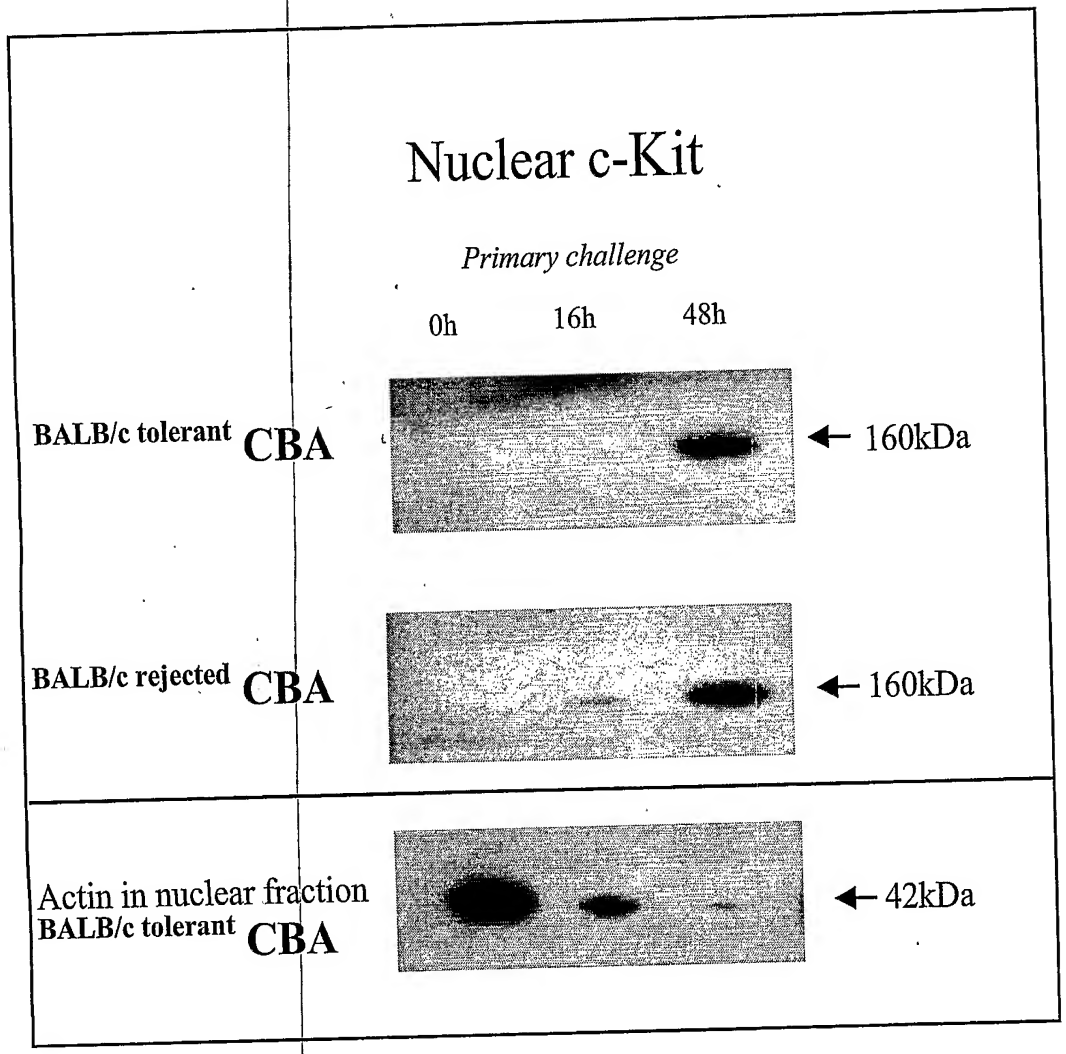

Nuclear c-Kit
Primary challenge
0h
16h
48h
BALB/c tolerant CBA
160kDa
BALB/c rejected CBA
160kDa
Actin in nuclear fraction
BALB/c tolerant CBA
42kDa

Table 1: Cytokine release following ex vivo stimulation of CBA spleen cells from BALB/c tolerant, or BALB/c rejected, mice challenged with irradiated BALB/c spleen cells

		Cytokine (pg/ml) in culture medium									
		INFγ		**IL2**		**IL4**		**IL10**		**IL12p70**		**IL13**	
Primary stimulation													
responder.: stimulator.	*Time*	TOL*	*REJ***	TOL	*REJ*	TOL	*REJ*	TOL	*REJ*	TOL	*REJ*	TOL	*REJ*
CBA : BALB/c	**0 h**	0	*0*	0	*0*	0	*0*	0	*407 ****	0	*0*	0	*82*
	16 h	0	*720*	0	*51*	18	*0*	293	*593*	0	*0*	0	*94*
	24 h	0	*1171*	11	*50*	7	*20*	358	*685*	13	*0*	0	*94*
	48 h	212	*4540*	158	*148*	14	*0*	538	*854*	0	*0*	50	*140*
Secondary stimulation @120 h													
CBA : BALB/c	**120 h**	1378	*8551*	364	*239*	60	*114*	733	*877*	0	*34*	127	*219*
	121 h	1456	*7944*	411	*227*	74	*0*	838	*457*	0	*13*	121	*151*
	123 h	1859	*8587*	293	*190*	73	*0*	899	*503*	0	*18*	143	*266*
	126 h	1569	*7506*	303	*172*	73	*0*	749	*492*	0	*26*	118	*152*
	144 h	5179	*14531*	137	*115*	135	*82*	1453	*853*	0	*32*	451	*458*
Self-self controls: Time													
CBA : CBA	**16 h**	0	*322*	0	*50*	0	*81*	365	*305*	nd	*19*	nd	*278*
	24 h	0	*333*	16	*31*	0	*116*	403	*394*	nd	*35*	nd	*132*
	48 h	0	*371*	111	*45*	40	*163*	445	*421*	nd	*66*	nd	*118*
	121 h	723	*109*	130	*51*	119	*93*	615	*476*	nd	*52*	nd	*146*

* *TOL: CBA BALB/c tolerant responder spleen cells*

** *REJ: CBA BALB/c rejected responder spleen cells*

*** *A positive reading of IL10 at zero hour was reproduced in a repeated ELISA*

Table 2a:

LIF release following allo-activation of BALB/c tolerant, or BALB/c rejected, CBA spleen cells.

		LIF (pg/ml) in culture supernatant		
responder : stimulator	*Time*			
Primary stimulation		TOL[a]	*REJ*[b]	*REJ*[c]
CBA : BALB/c	**0 h**	0	*0*	*0*
	48 h	0	*0*	*0*
Secondary stimulation @120 h				
CBA : BALB/c	**120 h**	320	*22*	*ND*
	121h	250	*20*	*60*

a *TOL: CBA BALB/c heart tolerant responder spleen cells*

b *REJ: CBA BALB/c skin rejected responder spleen cells*

c *REJ: CBA BALB/c heart rejected responder spleen cells*

Table 2b.

Cloned Treg produce high levels of Leukaemia Inhibitory factor (LIF) in culture.

		LIF pg/ml	**IL10** pg/ml	**INFγ** pg/ml
TH1 (R2.2) [a]	*resting* [b]	2	0	**1,493**
	activated [c]	5	791	**2,077**
TH2 (R2.4) [a]	*resting* [b]	70	**1,168**	0
	activated [c]	260	**2,084**	430
Treg (D1) [a]	*resting* [b]	**420**	**1,580**	0
	activated [c]	**440**	**2,067**	877

a TH1, TH2, and Treg, CD4+ T cell clones, and their respective culture conditions, are as detailed in reference (27).

b After 14d in culture, supernatant from each clone was harvested and cryo-preserved at -20^{o}C until assay.

c Freshly washed clones were stimulated for 18h with immobilised anti-CD3 and anti-CD28 as described in ref (13)

p=7

Transplantation Tolerance: Gene expression profiles comparing allo-tolerance *versus* allo-rejection

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Ab)ct

In mice, infectious regulatory tolerance is inducible by CD4/CD8 blockade in recipients of vascularised heart grafts. Once established, this transplantation tolerance is robust and isolated tolerant spleen cells show powerful immune regulatory properties, being able to impose donor-specific allo-tolerance upon fully immune competent na ve recipientso. Using *BALB/c-tolerant*CBA [H-2^k] mice, we analysed spleen cell responses to donor (BALB/c [H-2^d]) antigen at a series of time points and in comparison with an identical *ex vivo* series of *BALB/c-rejected*CBA spleen cells (S.M.M. et al, manuscript submitted). The key fetaure of rejection was rapid IFNγ release. In contrast, IFNγ in tolerance was low and less than that released in response to third party antigen (C57Bl10 [H-2^b]). Positive markers of primed tolerance were high expression of STAT3 and c-kit, and release of LIF. Here we present a compound comparison of four gene arrays (tolerance *versus* rejection, at 48h, and at 123h) where a relatively small number of differentially expressed genes occured. In rejection, there was a strong progressive amplification of IFNγ and granzyme B mRNAs. In tolerance, both Emk and axotrophin were upregulated at 123h. Mice lacking Emk develop auto-immune disease (Hurov et al, Mol Cell Biol, 2001). Mice lacking axotrophin show abnormal axonal migration during development (Lyons et al, in press). Taken together, our results suggest a link between developmental regulation and immune regulation, and highlight a possible role for axotrophin in regulatory tolerance.

Materials and Methods

Generation of BALB/c-primed CBA mice.

CBA mice ($H2^k$) of 10 - 12 weeks of age received a fully mismatched, vascularised BALB/c ($H2^d$) heart graft to the neck, using the technique described by Chen [Chen, Z.K., Cobbold, S.P., Waldmann, H. & Metcalfe, S.M. Amplification of natural regulatory immune mechanisms for transplantation tolerance. *Transplantation* **62**, 1200-1206 (1996)]. Tolerance was generated by a 21 day course of alternate day therapy using blocking mAbs to CD4 and CD8 as previously described [Chen, Z.K., Cobbold, S.P., Waldmann, H. & Metcalfe, S.M. Amplification of natural regulatory immune mechanisms for transplantation tolerance. *Transplantation* **62**, 1200-1206 (1996)]. *BALB/c-tolerant*CBA spleen cells from tolerant recipients were isolated at least 100d after grafting for *ex vivo* analyses. For comparison, untreated CBA mice, 10 - 12 weeks of age, were grafted either with BALB/c tail skin which rejected by day 10, or with a BALB/c heart which rejected on day 7. The *BALB/c-rejected*CBA spleen cells were collected at 14d for *ex vivo* analyses. All procedures were carried out according to Home Office licence under the Animals (Scientific Procedures) Act 1986, UK.

Ex vivo cultures

Culture conditions have been described in detail elsewhere [Metcalfe, S.M. & Moffatt-Bruce, S.D. An *ex vivo* model of tolerance *versus* rejection: Comparison of STAT1, STAT4, STAT5 and STAT6. *Clin. Chem. and Lab.Med.* 38, 1195 - 1199 (2000)]. Briefly, responder spleen cells were obtained from either *BALB/c-tolerant*CBA, or *BALB/c-rejected*CBA, mice, and the tolerant and rejected cell populations were stimulated *ex vivo* by irradiated BALB/c spleen cells (donor antigen), using $4x10^7$ responders to $6x10^7$ stimulators in a total of 10ml growth medium supplemented with 10% FCS. After 48h, one flask each of tolerant and rejected spleen cells were removed for total RNA preparation. A second pair of flasks (one tolerant, one rejected) were boosted with a further $7x10^7$ stimulator spleen cells at 120h, and then harvested at 123h. At harvest, cells were collected onto ice, with any adherent cells being included following brief treatment with 0.25% trypsin. After resuspending the cells to homogeneity, a 1.5ml aliquot was removed for RNA extraction. After washing in ice cold 0.1% BSA/PBS, the cells were collected into sterile 15ml Falcon centrifuge tubes and pelleted at 1600rcf for 5min at +4°C.

Supernatant was discarded and the tube wiped clear of supernatant residue prior to resuspending the cells in pre-cooled Trizol reagent, vortexed, and then immediately stored at —80C. One ml Trizol was used per $6x10^6$ cells.

RNA isolation.

Samples were brought to room temperature and kept for 10 minutes before addition of 1ml chloroform and vortexing to an emulsion. After 15 minthe samples were centrifuged at 1600rcf for 10min at 40C. The upper phase was transferred to RNA-ase-free Eppendorff tubes in 400 l aliquots and an equal volume of isopropanol added. After gentle mixing and standing for 15 min, the samples were centrifuged at 13,000g at 40C for 10 min. The supernatant was removed and discarded. The RNA pelletwas washed in 350 l of 75 ethanol and sedimented at 7500g fo 5min at 40C. The supernatant was aspirated and the pellet air dried for 20 min. The aliquoted RNA pellets were collected together for each sample by dissolving and serial transfer of 50 l DH_2O; a second 50 l was used to serially collect washings from each tube, giving a final total sample volume of 100 l in DH_2O. This was stored at —80oC until transfer to the MRC HGRC at Hinxton Hall for customer service preparation of cRNA and array using Affymetrix U74 chips by standard methodologies.

Gene Array.

Analyses of the combined arrays was prepared using dChip software [Wong, C.U.W.H., PNAS USA, 98, 31, 2001].

RESULTS

Combined 48h and 123h arrays of the matched tolerant and rejected samples pairs gave 129 genes showing differential expression. To identify those genes that showed a biased expression in either tolerance, or in rejection, the results were ranked in three ways: those genes showing a positive shift from 48h to 123h (Table 1); those genes with high expression at 123h (Table 2); and those genes (tolerant) that showed a positive shift, whilst the rejection counterpart showed a negative shift from 48h to 123h (Table 3).

Of the genes that increased in expression from 48h to 123h, 10 were in the tolerant cultures with increases ranging from 1.71 fold to 4.00 fold. Expression of the same genes in the rejection response showed either no increase in expression or a decrease in expression (Table 1(a)). Of particular note was axotrophin, a newly discovered stem cell gene ; cyclin B2, associated with the cell cycle and cellular migration ; histone H2A-X that may play a role in chromatin remodelling; and ELKL motif kinase, also known as Erk, required to regulate the immune response and protect against auto-immunity . Table 1(b) shows the 5 genes that increased in expression in rejection. Again this increase was specific to rejection, with the exception of granzyme B with a twofold increase in both tolerance and rejection; however, the actual levels of granzyme B mRNA were six times greater in rejection than in tolerance. The 12-fold increase in INFγ mRNA in rejection was in accord with our previous findings of high INFγ protein release in these cultures.

Of those genes that showed high expression at 123h, within the context of the four arrays, 15 were in the tolerant set (Table 2(a)) and included axotrophin. In rejection, 13 genes are ranked in order of expression level in Table 2(b) with

gra me B and INFγ being the highest. This analytical approach therefore showed correlation with phenotype with respect to granzyme B and INFγ, and again placed axotrophin as being associated with tolerance, although the actual expression level was not great. A further analysis was made, identifying those genes that showed increased expression in tolerance whilst showing a decreased expression in rejection (Table 3). This revealed Histone H2A-X, involved in chromatin structure and remodelling ; ELKL motif kinase ; splicing factor 3b subunit 1 (SF3b-155), acting as part of the mRNA splicing complex and probably involved in exon removal; and cyclin B2, a regulator of the cell cycle and also involved in cellular migration when complexed with cdc2..

DISCUSSION

TABLE 1: Genes showing increased expression (48h *versus* 123h)

Gene	Accession Number	Tolerance: Fold increase	*Rejection: Fold increase*
TOLERANCE			
Dual specificity phosphatase 1	X61940	**4.00**	*0.99*
BCL2-like 11	AA796690	**3.11**	*1.15*
*Axotrophin**	AW212859	**2.9**	*1.00*
H2A histone family, member X	M33988	**2.22**	*0.46*
Interferon stimulated protein (20kDa)	AW122677	**2.21**	*0.95*
Chemokine (C-C) receptor 6	AJ222714	**2.02**	*0.95*
Cyclin B2	X66032	**2.01**	*0.59*
Paneth cell enhanced expression	U37351	**2.0**	*0.98*
Splicing factor 3b, sub-unit 1, 155kDa	A1844532	**1.93**	*0.59*
*ELKL motif kinase***	X70764	**1.71**	*0.63*
REJECTION			
Interferon gamma	K00083	0.69	***11.98***
Glutaryl CoA dehydrogenase	U18992	1.20	***5.10***
CD3 antigen, gamma polypeptide	M18228	1.23	***3.22***
Interleukin 1 receptor antagonist	L32838	1.00	***2.57***
Granzyme B	M12302	2.07	***2.52***

TABLE 2: Genes showing high expression at 123h within the context of the four arrays

Gene	Accession Number	Expression level @ 123h
TOLERANCE		
β-2 microglobulin	X01838	9047
Ring Finger protein 10	AB026621	4127
CD53 antigen	X97227	3927
Guanylate nuceotide binding protein 1	M55544	1005
Spermidine spermineN1 acyl transferase	L10244	1002
Glycoprotein 49A	M65027	975
Chemokine (C-C) receptor 6	AJ222714	972
BCL2-like 11	AA796690	752
Paneth cell enhanced expression	U37351	753
EST	AW047461	744
Chemokine (C-C motif) ligand 9	C-U49513	593
EST	A1060627	562
Dual specificity phosphatase 1	X61940	536
Expressed Sequence AU021774	A1854141	438
*Axotrophin**	AW212859	416
REJECTION		
Granzyme B	M12302	6766
Interferon gamma	K00083	3103
Metallothionein 2	KO2236	1952
Lectin, galactose binding, soluble 1	X15986	1887
RNA binding motif protein 3	AB016424	1725
Acidic nuclear Phosphoprotein 32 family, member B	A1842771	1665
Glutaryl-Coenzyme A dehydrogenase	U18992	1350
STAT3	U08378	1026
STAT5A	AJ237939	988
Calcylcin	X66449	856
CD3 antigen pyrophosphate	M18228	517
IL1 receptor antagonist	L38838	511
Exp Sequence AU044919	X67210	356

Ta 3. Genes showing increases in expression in tolerance *and* decreased expression in rejection

Gene	Accession Number	Gene description
H2A histone family, member X	M33988	Chromatin remodelling (Bassing; Bruno)
*ELKL motif kinase***	X70764	Immune regulation ((Hurov)
Splicing factor 3b, subunit 1, 155kDa	A1844532	RNA splicing, intron removal (Horie)
Cyclin B2	X66032	Cell cycle; cell migration (Manes)